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The Bone Morphogenetic Protein Family and Osteogenesis

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ABSTRACT The BMPs (bone morphogenetic proteins) are a group of related proteins originally identified by their presence in bone-inductive extracts of demineralized bone. By molecular cloning, at least six related members of this family have been identified and are called BMP-2 through BMP-7. These molecules are part of the TGF-beta superfamily, based on primary amino acid sequence homology, including the absolute conservation of seven cysteine residues between the TGF-betas and the BMPs. The BMPs can be divided into subgroups with BMP-2 and BMP-4 being 92% identical, and BMP-5, BMP-6, and BMP-7 being an average of about 90% identical. To examine the individual activities of these molecules, we are producing each BMP in a mammalian expression system. In this system, each BMP is synthesized as a precursor peptide, which is glycosylated, processed to the mature peptide, and secreted as a homodimer. These reagents have been used to demonstrate that single molecules, such as BMP-2, are capable of inducing the formation of new cartilage and bone when implanted ectopically in a rodent assay system. Whether each of the BMPs possesses the same inductive activities in an animal is the subject of ongoing research. Based on the chondrogenic and osteogenic abilities of the BMPs in the adult animal, the expression of the mRNAs for the BMPs has been examined in the development of the embryonic skeleton by in situ hybridization. These studies demonstrate that the BMP mRNAs are spatially and temporally expressed appropriately for the proteins involved in the induction and development of cartilage and bone in the embryonic limb bud. Furthermore, primary preparations of limb bud cells respond to BMP-2, as do several cell lines of the osteoblastic lineage. In addition to expression in the skeletal system, various of the BMP mRNAs are expressed in distinct tissues, suggesting additional roles during development.

Key Words: BMP, TGF-beta superfamily, Bone

INTRODUCTION

Similar to the process described by Anita Roberts for TGF-beta, the discovery of the Bone Morphogenetic Proteins (BMPs) came from a search for the molecules responsible for a particular activity. In this case, it had been known for about 30 years that protein extracts from bone implanted into animals at non-bone sites will induce the formation of new cartilage and bone tissues (Urist, 1965). In this system, the sequence of events recapitulates the process of bone formation seen during embryonic long bone development (Urist et al., 1979; Reddi, 1981). This process is known as endochondral ossification, in which you observe a cartilage intermediate rather than intramembranous bone formation where bone is formed directly from mesenchyme. The identification of these osteoinductive molecules would allow their use as therapeutic treatments for a variety of bone defects, including nonunions, fractures, and periodontal disease.

Purification of Bovine BMP and Cloning of the Human BMPs

BMP activity was purified from bovine bone using ectopic bone induction in rats as the assay system (Sampath and Reddi, 1981), which is a reconstitution assay. Bone can be seen as consisting of three different components: 1) a mineral component which gives it its structural integrity, 2) a collagenous matrix component, and 3) a growth factor component which contains the BMP activity. The growth factor component can be extracted after the bone has been demineralized. To follow the BMP through its purification, the protein was assayed by reconstituting the fractions with rat bone collagenous matrix from which the endogenous BMP activity has been removed, and testing the effect of these reconstituted fractions by implantation subcutaneously in a rat. The implants were left in the animal for 7–14 days and then removed and examined histologically for the presence of newly formed cartilage and bone tissue. Thus, this assay system, which defines the BMP activity, is the result of a complex series of cellular events. At early times the implant area is infiltrated by the undifferentiated cell types. By days 4–7 these undifferentiated mesenchymal cells differentiate into chondrocytes. The chondrocytes mature and calcify. Around day 10, the beginning of new bone formation is seen as the cartilage intermediate is removed. At very late times the tissue develops into normal remodeling bone tissue which is complete with osteoblasts in the process of forming bone and osteoclasts that are resorbing it.

Using this assay system, we purified BMP activity approximately 300,000-fold from bovine bone (Wang et

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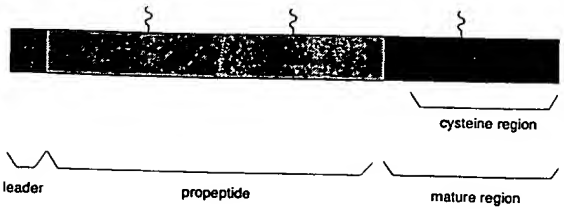

Protein	Additional names	Amino acid residues	Glycosylation sites (mature)
BMP-2	BMP-2A	396	1
BMP-4	BMP-2B	408	2
BMP-5		454	3
BMP-6	Vgr-1	513	3
BMP-7	OP-1	431	3
BMP-3	osteogenin	472	1

Fig. 1. Structure of the BMP proteins derived from cDNA clones. A generic BMP molecule is shown schematically with its secretory leader sequence, propeptide region, and carboxy-terminal mature region. Each of the BMPs contains N-linked glycosylation sites in both the propeptide and mature regions. The table below contains alternative nomenclature for the BMPs, the number of amino acids in the preprotein (primary translation products), and the number of potential N-linked glycosylation sites in the mature regions of the molecules.

al., 1988). While the yield is difficult to determine, there is probably about 20 μg of protein with bone inductive activity in about 10 kg of bovine bone. By assaying for BMP activity in proteins eluted from various gel electrophoresis systems, we determined that the BMP activity behaves in electrophoresis as a protein with a molecular weight of about 30,000 daltons, and the BMP protein is basic. However, gel electrophoresis of the 30,000 molecular weight protein under reducing conditions indicated the presence of multiple protein bands. We were not able to further purify the activity or determine which protein bands were responsible for the BMP activity because the activity is sensitive to reduction. Therefore, our strategy was to take the protein that we knew had BMP activity, digest it with trypsin, determine the sequences of the tryptic peptides, derive cDNA clones, express the cDNA clones, and determine which recombinant proteins in the mixture had osteoinductive activity.

From our cloning efforts we ended up with seven different proteins which we have named BMP-1 through BMP-7 (Wozney et al., 1988; Celeste et al., 1990). Six of the seven proteins are in the TGF-beta family (see Fig. 1). They are all secreted proteins with a hydrophobic leader sequence and a substantial propeptide region. Unlike TGF-beta, each of these proteins is glycosylated. These molecules are also known by alternative nomenclatures. For example, BMP-3 is the same as osteogenin (Luyten et al., 1989); BMP-6 is the human homologue of the murine Vgr-1 protein (Lyons et al., 1989; and BMP-7 has also been called OP-1 (osteogenic protein 1, Özkaynak et al., 1990).

The BMP Protein Family

The BMP proteins can be divided into subgroups based on the primary amino acid sequence in the mature regions of the molecule. BMP-2 and BMP-4 are quite closely related molecules, being 92% identical in the cysteine portion of the mature region. BMP-5, BMP-6, and BMP-7 form another subgroup with about 90% amino acid identity in the same region. These two subgroups are interrelated by about 60% amino acid identity, while BMP-3 is in a group of its own.

Comparing BMPs to other members of the TGF-beta superfamily, BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7 are most closely related to *Drosophila decapentaplegic (dpp)*. Because BMP-2 and BMP-4 are so closely related to *dpp*, it is quite clear that these molecules are its mammalian homologues. BMP-5, BMP-6, and BMP-7 are probably the mammalian homologue to the newly discovered 60A or *Vgr*/60A gene. The next most closely related protein to the BMPs is Vg1, a protein of unknown function from *Xenopus laevis*. After Vg1, the next most closely related protein is activin-A. The BMPs are also distantly related to TGF-beta 1, 2, and 3.

When BMP-2 was expressed as a recombinant protein in CHO cells, the transfected cells secreted a variety of BMP-2 molecules, the most predominant being a homodimer of the mature molecule. In addition, the clipped propeptide region is found secreted into the culture medium. The BMP-2, BMP-4, and BMP-7 propeptides are secreted as monomers because they contain no cysteine residues to allow intermolecular crosslinks. We also observed small amounts of partially processed dimers of each of the BMPs.

Osteogenic Activity of BMP in the Rat Ectopic Assay

We first assayed the recombinant BMP proteins in the rat ectopic assay system to compare their activities with bone-derived BMP (Wang et al., 1990). Initially we used rat demineralized inactive bone matrix as a carrier for BMP-2, placed rhBMP-2 combined with this matrix subcutaneously into the rat, and left the implants for various times before examining them histologically. In the control implants, the carrier matrix particles elicited migration of occasional undifferentiated mesenchymal cells into the site. The inclusion of BMP-2 resulted in much more cell invasion as well as recognizable chondrogenesis by 5 days. At 7 days there was some cartilage remaining in the implants, some new bone formation, and the matrix was being resorbed. After about 21 days we were left with a small ossicle of mineralized bone, a layer of osteoblasts laying down bone, and a mature fatty marrow. From these studies it was evident that BMP-2 is sufficient to induce the formation of bone in vivo, and that the one molecular species rhBMP-2, has all the activity of bone-derived BMP in this assay system.

To further quantitate the action of BMP-2 we established a scoring system on a sliding scale of 0–5 in

which the score 0 indicates no cartilage or bone present, and a score of 5 is given when the entire implant is composed of cartilage or bone. Using this scoring system, we did a dose-response and time course study. These results showed that cartilage formed at earlier times and then was replaced by bone at later times. It was possible to decrease the time required to observe bone formation by increasing the amount of rhBMP-2 in the matrix. High concentrations of rhBMP-2 resulted in concurrent cartilage and bone formation at early times after protein implantation.

A comparison of the primary sequences of BMP-2 and BMP-4 shows that these proteins are almost identical in the cysteine domain. However, the two proteins are quite different in the amino terminal domain despite the fact that they are both very basic in this region. Both proteins are active in the rat ectopic system (Hammonds et al., 1991), although addition of about twice as much BMP-4 was required for the same amount of bone formation as seen with doses of BMP-2. The time course of BMP-4-induced bone formation was slightly slower than for BMP-2.

BMP-5 also has the same osteoinductive effect as BMP-2 and BMP-4, but the time course of the osteoinductive response is significantly delayed when compared with that of BMP-2 (Cox et al., 1991; D'Alessandro et al., 1991). With BMP-5 implantation there is no observed bone formation until day 7. Furthermore, significantly more BMP-5 than BMP-2 is needed to observe the same amount of bone formation (Fig. 2). The same levels of bone formation as achieved by BMP-2 are never achieved with BMP-5 at either a comparable dose or a comparable time.

To summarize, bone-derived BMP activity is due to a set of proteins related to TGF-beta. In our hands we would say it is due to the proteins BMP-2 through BMP-7. Others have reported purifications where they observe activity with a combination of BMP-2 and BMP-7 (Sampath et al., 1990, 1991), or that BMP-3 is the sole BMP activity (Luyten et al., 1989). Individual proteins of this family of BMP molecules are sufficient alone for induction of bone formation in vivo. This has been demonstrated for BMP-2, BMP-4, BMP-5, and BMP-7. However, conclusive data has not yet been reported for BMP-3.

Induction of Bone in the Segmental Defect Model

We have initiated some studies to determine whether BMP proteins, in particular BMP-2, will be therapeutically useful in the human. Specifically, we wanted to answer the following questions: First, will rhBMP-2 induce bone formation at a bony site and produce new bone that will integrate with the preexisting bone to form a functional union? Second, will rhBMP-2 be able to induce bone formation in higher animals? For example, while the data above demonstrates that the BMPs will induce bone at an ectopic site in a rat, it is known that the rate of bone formation in rats is significantly higher than in humans.

One study was done using a rat femoral defect model in collaboration with Alan Yasko and Joseph Lane at the Hospital for Special Surgery in New York (Yasko et al., 1991). In this model a 5 mm femoral defect was created and fixed using a polyethylene plate attached with Kirshner wires; rhBMP-2 was implanted in the defect in a bed of inactive bone matrix. The experimental groups in this study were treated with a low or high dose of recombinant BMP-2. In all the studies the carrier system was allogeneic, guanidine-extracted bone matrix. The matrix alone was used as the control. Bone growth was evaluated by three criteria: 1) weekly autoradiographs, 2) histology at the end of the study, and 3) biomechanical studies. Without treatment, a defect of this size will proceed to become a nonunion in 100% of cases. Thus, in the controls, there was a small amount of growth at both ends of the defect but insufficient osteoconduction to fill the gap. By 4.5 weeks, in defects treated with the higher doses of rhBMP-2 there was already sufficient bone formation to be defined as a union by an orthopedic surgeon examining the radiographs. The results of biomechanical studies in which the femurs of four animals implanted with rhBMP-2 were torque-tested to determine the strength of the bone showed that the bone was quite strong compared to the untreated femurs. The presence of the holes through which the Kirshner wires were threaded weakened the BMP-treated femurs such that they could not be as strong as the untreated femur.

In a second study, sheep were used as the experimental animal (Gerhart et al., 1991). A similar experimental design was used as for the rat in which BMP adhered to matrix or matrix alone was implanted in a 2.5 cm defect in the sheep femur held together by a single metal plate. This study was done in collaboration with Dr. Tobin Gerhart and Dr. Carl Kirker-Head at Tufts Veterinary School. Eleven of the twelve sheep completed the study. In one sheep there was a failure of the fixation plate. The matrix control failed to heal and by the end of the study the region was quite grossly mobile and had developed a pseudoarthrosis. We also performed autologous bone grafting as a positive control. This is the current preferred therapy that would be used to treat a defect like this in humans. The autologous graft and the BMP-2-treated defect showed union by 12 weeks. In these animals the implantation sites of the femurs were grossly quite rigid. The results of biomechanical studies showed that the BMP-2-treated bone and the autologous graft were comparable to the contralateral control. By contrast, the bone with no implant or the bone treated with matrix alone showed no bone union. Histologically a large amount of bone formation could be observed in the BMP-2-treated defect with some remodeling already evident in which a cortex with a marrow cavity was being produced.

Our third study was done in collaboration with Dr. Dean Toriumi and used a dog mandibular defect model (Toriumi et al., 1991). The dog is considered to have bones that most closely approximate those of humans in size and remodeling sequence. In addition, this site pro-

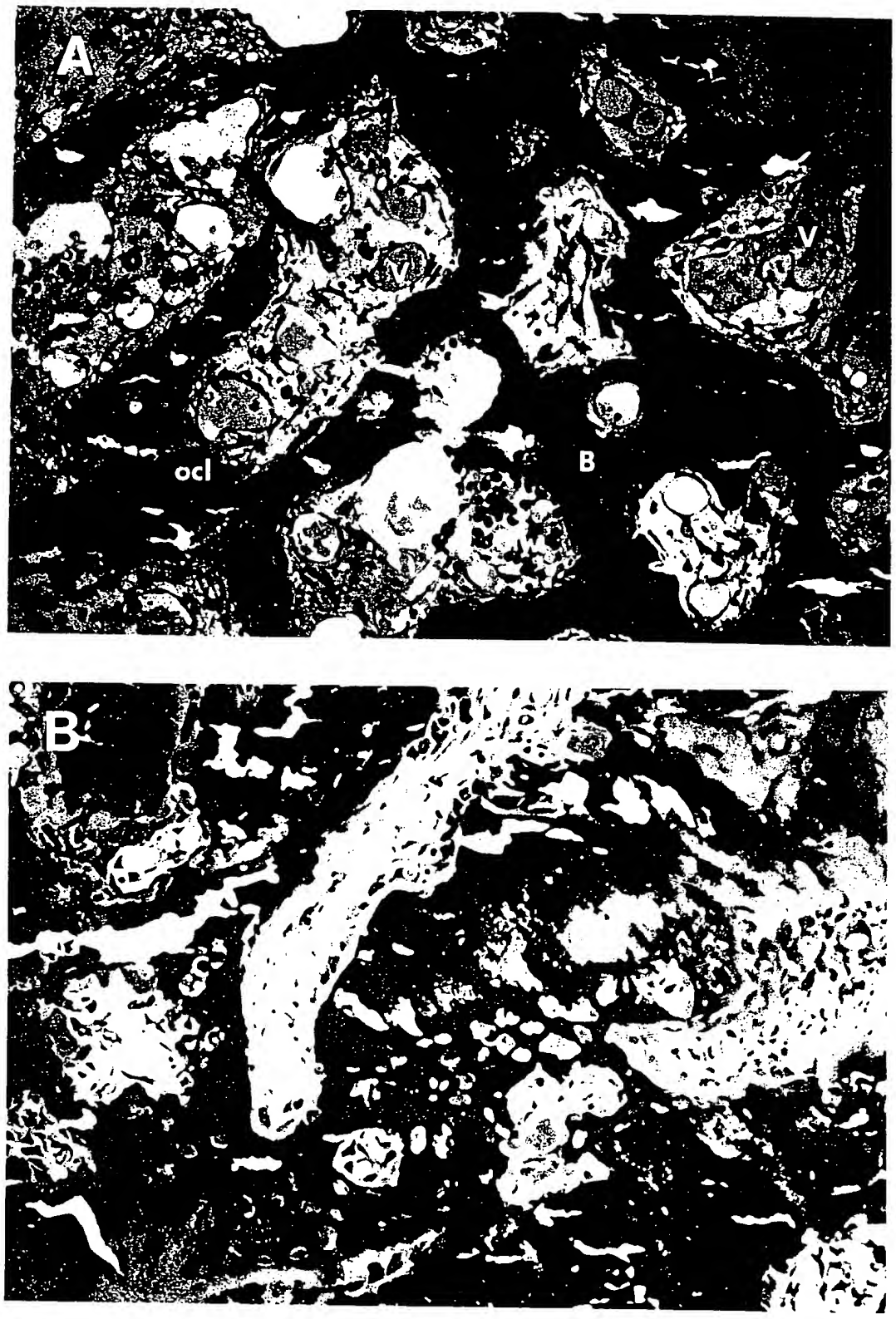

Fig. 2. Comparison of activities of rhBMP-2 and rhBMP-5: 10 μg of rhBMP-2 (A) or rhBMP-5 (B) were implanted subcutaneously in rats for 15 days. Implants were removed and processed for histology. C, calcified cartilage; ob, osteoblasts; M, unresorbed carrier matrix particles; ocl, osteoclast; V, blood vessel; mw, bone marrow; B, newly formed bone.

vides information on the efficacy of BMP-2 bone induction in a bone which is formed through the intramembranous sequence during embryogenesis, rather than the endochondral process that we associate with long bones. Three centimeter defects were made in dog mandibles. The mandibles were fixed with a metal plate, and then implanted with matrix alone as a negative control, matrix impregnated with BMP, or left empty. Filling of the defect with matrix alone resulted in fibrosis in the defect area, the same as leaving the defect empty. With BMP-2, the defect was totally filled with bone after 3 months. By this time the bone was already significantly remodeled.

To summarize the results of these studies, we have found that rhBMP-2 induces bone in all the models we investigated; in the rat ectopic bone formation assay and in the rat, sheep, and dog segmental defect models. It is interesting that the amount of BMP-2 needed and the time required to produce bone is similar in all these animals even though the sizes of the animals and their metabolic rates are significantly different. The rate-limiting parameter may be the volume of bone to be replaced; that is, the amount of time it takes the responsive cells to penetrate through the implant site and respond to the BMP-2.

The events observed in response to BMP in vivo are quite complex. It is possible that the function of the BMPs is to induce the initial step of the differentiation of mesenchymal cells into chondrocytes and then in vivo other growth factors, cells, and processes become involved to yield the cascade of bone formation. There is also some circumstantial evidence that BMP-2 may affect multiple stages in the bone formation process. In most studies the sequence of events observed in response to BMP implantation in vivo was differentiation of mesenchyme, chondrogenesis, hypertrophy, maturation of the cartilage, removal of hypertrophic cartilage, and then osteogenesis. However, with large amounts of BMP, osteogenesis can be seen concurrently with chondrogenesis. We were therefore interested to determine whether rhBMP-2 could affect the osteogenic phenotype of cultured cells.

The calvarial-derived, multipotential C26 cells are capable of differentiating into osteoblasts, adipocytes or muscle cells. In a collaboration with Dr. Suda and Dr. Yamaguchi, the effects of rhBMP-2 on these cells were examined (Yamaguchi et al., 1991). At large doses of rhBMP-2, we observed induction of alkaline phosphatase and a cAMP response to PTH, both markers of osteoblasts. In addition, the ability of these cells to differentiate into muscle decreased after exposure to rhBMP-2, as measured by the number of desmin-positive cells. Also, rhBMP-2 strikingly induced the expression of BGP mRNA. BGP is also called bone gla protein or osteocalcin and is probably the one specific marker of the mature osteoblast phenotype. The induction of osteocalcin was enhanced by the presence of 1,25 dihydroxy-vitamin D3.

Another osteoprogenitor cell compartment is the bone marrow stroma. W-20 cells are a multipotent mouse bone marrow stromal cell that is able to differentiate into osteogenic cells and adipocytes. Upon treatment with rhBMP-2, W-20 cells increased the expression of alkaline phosphatase (Thies et al., 1992). We have investigated numerous factors in this assay and have found no other factor or hormone which has been implicated in bone formation that will induce W-20 cell alkaline phosphatase production. In contrast, TGF-beta appeared to slightly inhibit alkaline expression by W-20 cells. BMP-5 also increased the production of alkaline phosphatase by W-20 cells, but the dose response was shifted such that more BMP-5 than BMP-2 was required for comparable enzyme induction. These results paralleled our observations on bone induction in vivo, i.e., in both cases, more BMP-5 than BMP-2 was required for a comparable effect. On the other hand, BMP-4 was more active than BMP-2 in the cell cultures, but slightly less active than BMP-2 in vivo. Trypsin-treated BMP-2 had a dose response that exactly overlaid that of BMP-4. This limited trypsin digestionof BMP-2 resulted in loss of the N-terminal region in which exists the major differences in sequence between BMP-2 and BMP-4, suggesting that the N-terminus of BMP-2 modulates its action. The N-terminus may inhibit the activity of BMP-2 by increasing its interaction with nonsaturable binding sites on the cell's surface or in the extracellular matrix, such that the concentration of BMP-2 available to its receptors is lower than its actual concentration.

Recently reported by Sampath and his colleagues are some studies of the responses of primary calvarial osteoblast-like cells to BMP-7 (also called OP-1; Knutsen et al., 1991). These cells, which are probably a mixture of osteoblast progenitors at different stages of differentiation, responded to BMP-7 with increases in collagen production, proliferation, and increases in various osteoblast characteristics such as alkaline phosphatase activity, PTH-stimulated cAMP production, osteocalcin synthesis, and mineralized nodule formation (BMP-7 in combination with ascorbate and betaglycerolphosphate).

So, from all these studies of cultured cells, it seems that the BMPs can stimulate cells to differentiate into the osteoblastic phenotype or cause them to increase the expression of osteoblastic phenotypic markers.

The Role of BMPs in Embryogenesis

The first evidence for a role of BMPs in embryogenesis is the realization that BMP-2 and BMP-4 are the mammalian homologues of the *Drosophila dpp*. From the results of powerful genetic analyses of Drosophila we know that *dpp* is responsible for either delivering positional information or interpreting it. Therefore BMP-2 and/or BMP-4 may have similar roles in mammalian development. The second line of circumstantial evidence for their role in mammalian development comes from the results of in situ localization of the various mRNAs for the BMPs during murine embryogenesis (Rosen et al., 1989; Lyons et al., 1989, 1990; Jones et al., 1991). Bone morphogenetic proteins 2 and

Fig. 3. Localization of BMP-4 transcripts in the developing mouse limb bud. Sections of 10.5 dpc mouse embryos were hybridized to a BMP-4 RNA probe labeled with [^{35}S]. Localization is evident in the apical ectodermal ridge.

4 were both found in the apical ectodermal ridge of the limb-bud very early in development (Fig. 3). The apical ectodermal ridge is required for positional information in the developing limb bud. Later in development BMP-2 mRNA was found in the interdigital mesenchyme of the limb located between the regions where the cartilaginous condensations will occur. BMP-2 mRNA expression is also observed in prevertebrae and in the tooth bud. Although in one report BMP-2 and not BMP-4 was seen in the developing tooth bud (Jones et al., 1991), others have observed that BMP-4 is also expressed in the developing tooth but with a different spatial distribution than BMP-2. Later in development, BMP-2 and BMP-4 are found in the more mature perichondrium, periosteum, and in odontoblasts. We have never seen BMP-3 in developing limbs or the skeletal system until very late in development when it appears in the periosteum. We also know that BMP-6 (Vgr-1) is not seen early in the developing limb but is seen later in the hypertrophic cartilage. Thus, BMP-6 may have a significantly different role from BMP-2 or BMP-4 in the developing skeleton.

Along with areas of bone formation, the BMPs were also found in many other regions of the developing embryo. For example, BMP-2 is found in the heart and in whisker follicles. BMP-6 is found in brain, spinal cord, and skin. BMP-3 is found in the brain. In many of these regions various TGF-betas are also expressed.

Because of our interest in the developing limb and the role of BMPs in this process, we were interested in whether the BMPs could affect the development of limb bud cells. Therefore we have isolated cell lines from the limb buds by taking mouse embryos at various stages, removing the limb buds,and immortalizing the cells in the limb buds using a retroviral vector with a c-*myc* gene and a neomycin resistance gene as a selectable marker. The neomycin resistant cells were selected and examined for their phenotype and their growth responses to BMPs. Most of the work to date has been on cells derived from 13-day mouse embryos. The pool of cells responded to BMP-2 with a substantial increase in alkaline phosphatase, which could be potentiated by the addition of retinoic acid. The pool also responded to BMP-2 with an increase in bone gla protein expression (Rosen et al., 1991). After treatment with BMP-2, the pool of immortal limb bud cells formed nodules which could be stained with Alcian blue, a stain specific for cartilage extracellular matrix proteins. Therefore these cells were capable of forming cartilage nodules, a process that was significantly increased by the addition of BMP-2. In micromass cultures densely staining cartilage nodules could also be observed with the addition of BMP-2. Both the number of Alcian blue staining nodules and the incorporation of radiolabeled sulfate into proteoglycans can be used to quantitate the chondrogenic response to BMP-2. Using these methods, there is both a dose and time dependent increase in expression of the chondrogenic phenotypes in response to BMP-2.

We have also derived individual clones from this pool of cells and they display a variety of phenotypes. For example, clone number 5 responded to BMP-2 with at least a 10-fold increase in alkaline phosphatase but showed no response to TGF-beta-1. This clone also responded with an increase in cAMP when treated with PTH. Thus, one can derive cells from embryonic mouse limb buds at times as early as 13 days of development that express markers of the osteoblast lineage.

To summarize what we know about the activities of rhBMP-2 in cultured cells: it increases expression of markers of the osteoblast phenotype in the MC3T3E1 and the C26 cell lines. The C20 cells, which represent more differentiated osteoblasts, show no increase in alkaline phosphatase but some increase in PTH-stimulated cAMP production. From these results it seems that the cells which are more responsive to the BMPs are the earlier osteoprogenitor cells rather than the more differentiated osteoblasts. The activity of BMP-2 on W-20 cells derived from the bone marrow stromal environment is to increase alkaline phosphatase, PTH-

stimulated cAMP production, and osteocalcin synthesis but not to increase proliferation. In the embryonic 10T1/2 cell line it has been reported that alkaline phosphatase and the PTH response increases after BMP treatment. Finally, the BMPs increase several parameters and characteristics of the chondrogenic lineage in the immortal embryonic limb bud cells.

In summary, BMP-2 activates cells that participate in bone repair. This has been demonstrated in vivo in a variety of species, as well as in cultured cells from both the osteoblastic and chondroblastic lineages. In adult animals, rhBMP-2 is capable of inducing the formation of new bone, and thus can successfully treat large bony defects. The BMPs can also increase the differentiated state of primary embryonic cells, indicating that the BMPs are involved in the development of cartilage and bone during embryogenesis.

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QUESTIONS AND ANSWERS

Q: *Can the bone matrix that you use be substituted by a synthetic polymer?*

A: That's a good question. Essentially all the studies that I discussed have been repeated using a defined collagenous or a synthetic matrix and BMP-2 works perfectly well with these matrices.

Q: *Have you observed any effect of TGF-beta-1 or TGF-beta-2 in vivo?*

A: We have done some studies with TGF-beta and really haven't seen any effect. I think it's complicated because it's quite possible that if you could somehow put BMP in at day 1 and then TGF-beta in at day 3 it might work well. Putting them in together at the beginning doesn't seem to have any different effect than BMP alone.

Q: *Do any of your limb bud cell lines respond to TGF-beta and not to BMP?*

A: The cell lines were selected on the basis of responses to BMPs, so that question has not really been addressed.

Q: *Do you need more recombinant than natural BMPs for full activity in vivo?*

A: That is an arguable point even within our group. I would say that you get a better response with the bone-derived BMP than with any single recombinant BMP.

Q: *Have you tested the effect of combinations of recombinant BMPs?*

A: We've done some combination studies and in these we have not seen anything other than an additive effect.

Q: *What concentrations of BMP did you require to observe differentiation of the primary limb bud cells?*

A: The responses of immortal and primary cells were seen at about 20–50 ng/ml BMP.

Q: *Have you tested the effects of BMP on osteoporosis in ovariectomized animals?*

A: These are difficult studies to do because the BMPs are cleared so rapidly from the circulation.

Q: *The dosage at which you see an effect in cultured cells is remarkably high for a factor that is present in remarkably low levels in vivo. These are also high levels when compared with effective concentrations of TGF-beta. Do you think there is something in vivo that potentiates the effects of the BMPs?*

A: That's a very difficult question to answer and is something I've thought a lot about. It is possible that there is something about the way it is presented to the cells in vivo compared with the presentation in culture that increases its potency. The effects of BMP-4 and trypsinized BMP-2 occur in a more reasonable range of concentrations. But these are still high compared with other growth factors.

Q: *Is there any evidence for massive degradation of the BMPs in culture?*

A: No, these are extremely stable molecules.

Q: *Does the healing of the segmental defect model in the dog proceed through a cartilaginous intermediate?*

A: We did not do histology at different time points so as to avoid using a lot of animals. But it is known that when you fracture that type of bone you repair it by an endochondral sequence. So I would guess that the BMP-induced bone healing does go through endochondral bone formation.